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(54) Title: METHOD FOR INHIBITING SMOOTH MUSCLE CELL PROLIFERATION AND OLIGONUCLEOTIDES FOR USE THEREIN

(57) Abstract

The present invention provides multi-G oligonucleotides that are capable of inhibiting smooth muscle cell proliferation. The multi-G oligonucleotides of the present invention can be used as nucleic acid therapeutic compounds to inhibit smooth muscle cell proliferation in arteries *ex vivo*, and more particularly against restenosis. The present invention further provides pharmaceutical compositions containing the multi-G oligonucleotides. A method for screening oligonucleotides for their ability to inhibit smooth muscle cell proliferation *ex vivo* is also provided.

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METHOD FOR INHIBITING SMOOTH MUSCLE CELL PROLIFERATION AND OLIGONUCLEOTIDES FOR USE THEREIN

Field of the Invention

This invention relates to the field of therapeutics, and in particular the field of nucleic acid therapeutics.

Background

One particular area of clinical significance that has recently become the object of nucleic acid therapeutic research is the area of restenosis. Restenosis involves the re-occlusion of blood vessels that have been at least partially cleared of occlusion and is, ironically, the result of the process employed to clear the occluded vessel(s) in the first place. For example, restenosis frequently occurs following balloon angioplasty or other catheter-based medical intervention (*e.g.*, close to 50% occurrence), but can also occur as the result of, *e.g.*, coronary by-pass surgery or any similar type of insult or injury to a blood vessel. The pathological process of restenosis is a hyperproliferative response that includes both smooth muscle cell proliferation and extracellular matrix accumulation. It is a widely held belief that the reclosure of an artery subjected to angioplasty is due primarily to a process akin to wound healing.

More specifically, it is believed that activated smooth muscle cells play a major role in the reclosure/injury response involved in restenosis, and that smooth muscle cell proliferation is a key element in th process. Activated smooth muscle c lls appear to proliferat and then migrate into the arterial lumen,

causing restenosis. Thus, inhibition of smooth muscle cell proliferation is believed to be a key element in the prevention of vascular restenosis following angioplasty or other insult to a blood vessel, and efforts toward preventing restenosis, or at least ameliorating the condition, have focused on methods for inhibiting smooth muscle cell proliferation. Many of these efforts have been focused within the biotechnology field, with varying levels of success having been reported in the areas of: (1) "traditional" approaches that require the interaction of proteinaceous substances directly with one or more of the proteins involved in smooth muscle cell proliferation; (2) "antisense" nucleic acid therapeutic compounds that interact at the genetic level with RNA (or sometimes DNA) involved in smooth muscle cell proliferation; and, (3) other nucleic acid therapeutic compounds which may interact at either the genetic level or at the protein level.

An example of the first type of approach is the use of a monomeric form of the normally dimeric platelet-derived growth factor (PDGF) molecule to inhibit restenosis, as reported in International Application No. PCT/US93/02612. In this case, a monomeric form of PDGF is provided as a therapeutic compound in excess over the naturally occurring dimeric molecule. In order to be effect against restenosis, the therapeutic compound must successfully compete with dimeric PDGF for binding sites (PDGF receptors) on smooth muscle cells that are necessary for cell growth to be stimulated by PDGF. Like traditional pharmaceutical drug approaches, this represents a macromolecular (protein/protein) interaction.

In contrast, true antisense interactions involve hybridization of complementary oligonucleotides

(hence, the term "antisense") to their selected nucleic acid target (*e.g.*, viral RNA or other undesired genetic messages) in a sequence specific manner such that the complex thus formed, either alone or in combination with other agent(s) (*e.g.*, enzymes, such as RNAse) can no longer function as a template for the translation of genetic information into proteins. An antisense oligonucleotide may be designed to interfere, for example, with the expression of foreign genes (*e.g.*, viral genes, such as HIV), with the aberrant expression of endogenous genes (*e.g.*, a normal gene that is aberrantly expressed as a mutated oncogene), or with the normal expression of endogenous genes whose response to specific stimuli may be undesired in certain instances, such as in the case of restenosis where smooth muscle cell proliferation is undesired at the site of angioplasty or by-pass surgery.

Because antisense-based nucleic acid therapeutics raise the possibility of therapeutic arrest of restenosis at the early replication and expression stage, rather than attacking the resulting protein at a later stage of smooth muscle cell proliferation (as in the case of therapeutic products requiring direct interaction with the protein), antisense oligonucleotides have been actively pursued as the premier therapeutic compounds for use in the prevention or amelioration of restenosis. In this regard, previous studies have reportedly demonstrated inhibition of restenosis in *in vitro* and *in vivo* models following the application of antisense oligonucleotides targeting the *c-myb* and *c-myc* genes, among others, which are believed to be involved in the proliferation of smooth muscle cells. *See, e.g.*, International Application No. PCT/US92/05305; Simons, *et al.*, *Nature*, *359*, 67-70 (1992) (human *c-myb* gene); Shi *et al.*, *Circulation*,

90(2), 944-951 (1994) (*c-myc* gene in pigs); Morishita *et al.*, *Proc. Natl. Acad. Sci. USA*, *90*, 8474-8478 (1993); Morishita *et al.*, *J. Clin. Invest.*, *93*, 1458-1464 (1993) (*cdc* 2, PCNA, *cdk* 2 in rats).

Other nucleic acid therapeutic compounds have sequences that may or may not be complementary to a target sequence, but can, nevertheless, prevent an undesired result by interfering with either: (1) the expression (*e.g.*, replication and/or translation) of the undesired genetic material; or (2) the required interaction(s) of proteins involved in the cellular pathway. Recently, the use of oligonucleotides having multiple contiguous guanine residues has been suggested for use in inhibiting the activity of viruses, in addition to inhibiting the activity of phospholipase A_2 and modulating the telomere length of chromosomes. *See*, International Patent Application No. PCT/US93/09297, wherein: (1) at least one series of four contiguous guanine residues or two series of three contiguous guanine residues were reported to inhibit herpes simplex virus (HSV) or phospholipase A2 activity; and (2) at least one series of four contiguous guanine residues was reported to inhibit cytomegalovirus, influenza virus or human immunodeficiency virus (HIV).

The mechanism by which oligonucleotides having multiple contiguous guanine residues inhibit the various viral and enzymatic activities reported in the literature is not known, although it has been postulated that the action of these oligonucleotides against the HIV virus involves interaction between a tetrameric structure formed by the contiguous guanine residues of the oligonucleotide with the V3 loop of the gp 120 protein associated with the HIV virus. Wyatt *et al.*, *Proc. Natl. Acad. Sci. USA*, *91*, 1356-1360 (1994). It

has subsequently been noted that the anti-*c-myb* antisense compounds used by Simons, *et al.*, *Nature, 359,* 67-70 (1992), also, coincidentally, contain four contiguous guanine residues. (*See,* Yaswen *et al.*, *Antisense Res. and Dev., 3,* 67-77 (1993), wherein caution is urged in observations of true antisense activity where oligonucleotides containing multiple contiguous guanine residues are employed.) However, the exact inhibitory mechanism of guanine-rich oligonucleotides remains unknown, and is surely not identical in all situations. Moreover, the absence of a known V3 loop on any of the proteins involved in smooth muscle proliferation suggests that guanine-rich oligonucleotides are not expected to be effective against restenosis other than in the case of a coincidental appearance of a region of contiguous guanine residues as a natural consequence of composition of the target sequence.

It would be desirable to have a nucleic acid therapeutic compound that is capable of inhibiting restenosis.

Therefore, it is an object of the present invention to provide oligonucleotides that are able to potently inhibit smooth muscle cell proliferation.

It is a further object of the present invention to provide a method for inhibiting smooth muscle cell proliferation.

It is a still further object of the present invention to provide a method for screening oligonucleotides for their ability to inhibit smooth muscle cell proliferation.

Summary of the Invention

The present invention provides multi-G oligonucleotides that are capable of inhibiting smooth muscle cell proliferation. Pharmaceutical compositions containing the multi-G oligonucleotides of the present invention are also provided. These pharmaceutical compositions are useful in the method of the present invention, wherein multi-G oligonucleotides are used as nucleic acid therapeutic compounds to inhibit smooth muscle cell proliferation in arteries *ex vivo*, and more particularly against restenosis. The present invention further provides a method for screening oligonucleotides for their ability to inhibit smooth muscle cell proliferation.

Brief Description of the Drawings

Figure 1 is a table of modified oligonucleotide sequences used in the examples.

Figure 2A is a graph showing the percent suppression of rabbit smooth muscle cell proliferation by various oligonucleotide sequences at a concentration of 10 μM.

Figure 2B is a graph showing the percent suppression of rabbit smooth muscle cell proliferation by various oligonucleotide sequences at a concentration of 30 μM.

Figure 2C is a graph showing the percent suppression of rabbit smooth muscle cell proliferation by various oligonucleotide sequenc s at a conc ntration of 60 μM.

Figure 2D is a graph showing the percent suppression of pig smooth muscle cell proliferation by various oligonucleotide sequences at a concentration of 15 μM.

Figure 3 is a graph showing the percent suppression of rabbit smooth muscle cells by multi-G modified oligonucleotides (versus control) at a concentration of 15 μM.

Figure 4 is a graph showing the percent suppression of rabbit smooth muscle cells by inosine-substituted modified oligonucleotides at a concentration of 30 μM.

Figure 5 is a graph showing the percent suppression of BrdU incorporation into rabbit smooth muscle cells by modified multi-G oligonucleotides *vs.* control at a concentration of 30 μM.

Figure 6 is a graph showing the growth response of rabbit smooth muscle cells following removal of modified multi-G oligonucleotides *vs.* control at a concentration of 30 μM.

Figures 7A, 7B and 7C are graphs showing BrdU incorporation by smooth muscle cells in rabbit ear arteries as determined by measuring the number of BrdU-labeled nuclei per mm^2.

Detailed Description of the Invention

As noted above, the present invention provides multi-G oligonucleotides that are capable of inhibiting smooth muscle cell proliferation. Th multi-G oligonucleotides of the present invention are comparable

in length to true antisense oligonucleotides, but have multiple contiguous guanine residues at one or more locations in the oligonucleotide. These multi-G oligonucleotides can be used as nucleic acid therapeutic compounds to inhibit smooth muscle cell proliferation *in vivo*, and more particularly to inhibit vascular restenosis following injury or insult to a blood vessel. A method for screening oligonucleotides for their ability to inhibit smooth muscle cell proliferation *in vivo* is also provided.

In order to aid in the understanding of the present invention, the following terms, as used herein, have the definitions designated below.

"Oligonucleotide" refers to a polymer of at least two nucleoside units, wherein each of the individual nucleoside units is covalently linked to at least one other nucleoside unit through a single phosphorus moiety. In the case of naturally occurring oligonucleotides, the covalent linkage between nucleoside units is a phosphodiester bond. Nevertheless, the term "oligonucleotide", as used herein, includes oligonucleotides that are modified (as compared to naturally occurring oligonucleotides) with respect to any one or more of the following: (1) the phosphodiester bond between nucleoside units; (2) the individual nucleoside units themselves; and/or (3) the ribose, or sugar, moiety of the nucleoside units.

Unless otherwise specified, the term "base" or "nucleobase" refers to a purine or pyrimidine, such as adenine, guanine, cytosine, thymine, inosine and uracil as well as modified forms of these bases, such as 5-methylcytosine and 5-propynyl pyrimidines.

Unless otherwise specified, the term "residue" refers to a particular base or nucleobase as well as the sugar moiety to which it is attached and the phosphate moiety (or corresponding moiety in the case of a modified oligonucleotide) as it exists in an oligonucleotide.

The term "contiguous" means next to or adjacent. Thus, in the case of an oligonucleotide, "contiguous" nucleic acid residues refers to nucleic acid residues that are adjacent each other in the oligonucleotide sequence.

The term "multi-G oligonucleotide" refers to an oligonucleotide having at least four contiguous guanine residues or at least two series of at least three contiguous guanine residues.

The term "poly-G oligonucleotide" refers to an oligonucleotide having only guanine residues.

"Nucleoside" refers to an individual monomeric nucleoside unit consisting of a base covalently bonded to the 1'-position of a 5-carbon sugar. The 5-carbon sugar will typically be a naturally occurring sugar such as deoxyribose, ribose or arabinose, but can be any 5-carbon sugar or modified form thereof, including but not limited to, 2'-fluoro-2'-deoxyribose or even carbocyclic sugars (where a carbon function is substituted for the oxygen atom in the sugar ring; *i.e.*, 6-carbon analog) or thiol sugars (where a sulfur atom is substituted for the oxygen atom in the sugar ring). Typically, the base will be linked to the sugar moiety at conventional positions, such as N9 of adenine, guanine and other purines or N1 of cytosine, thymine, uracil and other pyrimidines.

"Nucleotide" refers to a monomeric nucleoside unit further having a phosphorus moiety covalently bonded to the sugar moiety of the nucleoside at either the 3'- or 5'-position of the sugar.

A "modified internucleotide linkage" refers to any modification of the phosphodiester bond joining individual nucleoside units in naturally occurring oligonucleotides that improves activity of the oligonucleotide, such as by imparting nuclease resistance to the internucleotide linkage.

The term "modified oligonucleotide" specifically refers to an oligonucleotide having at least one modified internucleotide linkage.

The term "partially modified oligonucleotide" means a modified oligonucleotide wherein at least one but fewer than all internucleotide linkages are modified.

The term "fully modified oligonucleotide" means a modified oligonucleotide wherein all of the internucleotide linkages are modified.

"Target sequence" refers to the nucleotide sequence to which an oligonucleotide or a modified oligonucleotide is designed to hybridize. In the case of inhibitory oligonucleotides, the "target sequence" may be, but is not necessarily limited to, a naturally occurring messenger RNA coding for a viral protein, cancer related protein or other proteins involved in disease states or other undesired conditions.

Surprisingly, it has been found that the multi-G oligonucleotides of the pres nt invention strongly inhibit th proliferation of primary smooth

muscle cells both in cell culture *in vitro* and in arteries *ex vivo*. Although the anti-proliferative effect of the multi-G oligonucleotides of the present invention is sequence specific, it does not operate according to a true antisense mechanism. Specifically, it is believed that the multi-G oligonucleotides of the present invention act as inhibitors of smooth muscle cell proliferation by interacting directly with one or more of the proteins involved in the proliferative process. This is in marked contrast to the interaction at a genetic level (*i.e.*, oligonucleotide/oligonucleotide) that is required in the case of true antisense oligonucleotides and has been alluded to even in the instance of antiproliferative effects that have been observed in the case of (antisense) oligonucleotides that coincidentally contain multiple contiguous guanine residues. *See*, Yaswen *et al.*, *Antisense Res. and Dev.*, *3*, 67-77 (1993).

The multi-G oligonucleotides of the present invention appear to inhibit smooth muscle cell proliferation by blocking cells from re-entering the cell cycle after stimulation with growth factors such as by the addition of serum. Furthermore, it is clear that, at least at lower concentrations, the inhibitory effect of the multi-G oligonucleotides of the present invention against smooth muscle cell proliferation is not due to non-specific toxicity. Indeed, smooth muscle cells in culture look healthy and appear to undergo a normal proliferative response if the multi-G oligonucleotide is removed and fresh media is added.

Rather than being designed to be complementary to a designated portion of a target sequence (*i.e.*, in the manner of a true antisense oligonucleotide), the multi-G oligonucleotid s of the present invention are designed to have at least one series of at least four

contiguous guanine residues or at least two series of at least three contiguous guanine residues. However, it is preferred that the multi-G oligonucleotides of the present invention have at least two series of at least four contiguous guanine residues, it being understood that the two series of contiguous guanine residues may occur adjacent to each other in the oligonucleotide such that, in the preferred case of at least two series of a least four contiguous guanine residues, a series of at least eight contiguous guanine residues results.

It is more preferred that the multi-G oligonucleotides of the present invention have at least two series of at least six contiguous guanine residues, it again being understood that these two series of contiguous guanine residues may be placed adjacent each other in the oligonucleotide, yielding a series of at least twelve contiguous guanine residues. In fact, in a particular preferred embodiment of the present invention, the multi-G oligonucleotides of the present invention have at least about twelve contiguous guanine residues. Regardless of the particular configuration of the series of contiguous guanine residues in the multi-G oligonucleotide, it is preferred that the multi-G oligonucleotides of the present invention have at least one base other than a guanine on each of the 3'- and 5'-ends of the oligonucleotide.

It is somewhat surprising that multi-G oligonucleotides of the present invention having at least five contiguous guanine residues were found to be significantly more potent at inhibiting smooth muscle cells than oligonucleotides having only four contiguous guanine residues. This is in contrast to th t achings of the arli r not d International Patent Application No. PCT/US93/09297, which reported significant levels of

inhibition with four contiguous guanine residues, *albeit* in the context of targets other than smooth muscle cells (*i.e.* viruses, phospholipase A_2) and, undoubtedly, different mechanisms of action.

The lengths of the multi-G oligonucleotides of the present invention may vary to some degree, but will generally be from about 10 to about 30 nucleotides in length. It is preferred that the multi-G oligonucleotide be from about 14 to about 20 bases long, as longer oligonucleotides within the 10 to 30 nucleotide range may hybridize nonspecifically to other non-target sequences if the oligonucleotide is too long (*i.e.*, substantially longer than 20 nucleotides). It is more preferred that the multi-G oligonucleotide be an 18-mer. The optimal length of a multi-G oligonucleotide according to the present invention will be readily apparent to a person of ordinary skill in the art following the teachings presented herein. Generally speaking, an oligonucleotide length of about 12 to about 25 bases represents an oligonucleotide length that is consistent with other oligonucleotides designed for nucleic acid therapeutics and is a manageable length from a practical standpoint. It is understood, however, that shorter oligonucleotides are more commercially feasible. Thus, it will almost always be preferred to employ the shortest oligonucleotide exhibiting the maximum efficacy.

Generally speaking, poly-G oligonucleotides are less preferred than other multi-G oligonucleotides according to the present invention, because of handling difficulties inherent with these oligonucleotides. Specifically, poly-G oligonucleotides exhibit a tendency toward self-aggregation, likely due to the formation of tetrads, wherein four-stranded structures are formed as a

result of intra-oligonucleotide hydrogen bonding of guanine residues. Although tetramer formation has been suggested as the key phenomenon involved in the observed inhibitory action of multi-G oligonucleotides against the HIV virus (Wyatt *et al, Proc. Natl. Acad. Sci. USA, 91,* 1356-1360 (1994)), these tetrameric structures are not believed to be necessary for inhibition of smooth muscle cell proliferation according to the present invention.

It will ordinarily be preferred that the multi-G oligonucleotides of the present invention be modified oligonucleotides in order to enhance activity of the oligonucleotide. For example, specific activity of the multi-G oligonucleotide will be increased over time where the oligonucleotide has been modified to resist the various nucleases that are endogenous to a human or animal body. Because unmodified oligonucleotides are susceptible to nuclease degradation at both the 3'- and 5'-positions of the internucleotide linkages, attempts to impart nuclease resistance to therapeutic oligonucleotides have primarily been directed to modification of the internucleotide linkage, with success having been achieved primarily with respect to modification of the "non-bridging" oxygen atoms in the naturally occurring phosphodiester linkage. (*E.g.*, phosphorothioate-modified oligonucleotides having a single non-bridging oxygen substituted with a sulfur atom (U.S. Patent No. 3,846,402) and phosphorodithioate-modified oligonucleotides having both non-bridging oxygen atoms substituted with sulfur atoms (U.S. Patent No. 5,218,103).) More recently, stable oligonucleotides having a 3'-carbon linkage have also been reported. *See,* copending U.S. Patent Application, Serial No. 08/221,425.

It is preferred that the multi-G oligonucle tides of the present invention be modified by

having sufficient phosphorothioate, phosphorodithioate or 3'-carbon modified linkages to impart nuclease resistance. Although phosphorothioate and phosphorodithioate linkages are more preferred, it is important to note that sulfur-containing oligonucleotides such as these are known to bind to proteins, resulting in a level of non-specific activity, that may have an undesired non-specific level of inhibition at higher concentrations of these oligonucleotides.

The multi-G oligonucleotides of the present invention can be made according to any one of a number of methods routinely known in the art. Generally, oligonucleotides are synthesized using automated synthetic protocols requiring large pieces of equipment such as the DNA synthesizers sold by Applied Biosystems (Foster City, California) and exemplified in Example 1, below. Automated DNA synthesizers may also be obtained from a number of other commercially available sources. The preferred type and source of equipment will vary, depending on the quantity of oligonucleotide required for scale-up and other (*e.g.*, commercial) considerations. Modified linkages, including phosphorothioate and phosphorodithioate modified linkages, as well as the other modified linkages referenced above, can be introduced into the multi-G oligonucleotides by employing variations of the standard protocols for phosphodiester linkages, as taught in the art. Other means for synthesis of the multi-G oligonucleotides of the present invention may also be employed, although actual synthesis of the oligonucleotides will be apparent to one of ordinary skill in the art following the teachings of the present invention.

A method for screening oligonucleotides f r potential therapeutic use against smooth muscle cell

proliferation is also provided in accordance with the present invention. The screening method of the present invention simulates the occurrence of restenosis *in vitro* and initially requires the arrest of smooth muscle cells in G_0 growth phase. After the smooth muscle cells have been arrested, proliferation of the smooth muscle cells is initiated by serum addition in the presence of multi-G oligonucleotides. Multi-G oligonucleotides demonstrating greater than 50% inhibition at a concentration of less than or equal to 10 μm are believed to be effective for use against smooth muscle cell proliferation in arteries *ex vivo*, with oligonucleotides demonstrating greater than 50% inhibition at or below 2 μm concentrations being particularly effective. It is preferred to attach the smooth muscle cells to a solid support in conducting the screening method. It is also preferred to arrest the smooth muscle cells in G_0 growth phase by placing the smooth muscle cells in a starvation media after which proliferation of said smooth muscle cells is initiated by replacing said starvation media with normal growth media containing serum.

The present invention further contemplates a method for inhibiting smooth muscle cell proliferation comprising the administration of multi-G oligonucleotides to a human or animal subject. Preferred multi-G oligonucleotides for delivery to such human or animal subject include multi-G oligonucleotides having four contiguous guanine residues and multi-G oligonucleotides having two series of three contiguous guanine residues. In the latter case, it is preferred that the two series of three contiguous guanine residues be separated by a single residue other than a guanine residue. Other preferred multi-G oligonucleotides for

use in the method of the present invention include the preferred multi-G oligonucleotides previously described.

The multi-G oligonucleotides of the present invention are preferably delivered locally to a site of insult or injury to a blood vessel that is, by virtue of such injury or insult, at risk of restenosis. Localized delivery of the multi-G oligonucleotides can be accomplished *via* direct injection at the desired site or can be delivered using an infusion pump. Preferably, the multi-G oligonucleotides will be combined with a pharmaceutically acceptable carrier and delivered by way of a catheter, stent, or other implantable device. In this way, the delivery of the multi-G oligonucleotides can be incorporated into a device used during the particular medical intervention procedure (*e.g.*, angioplasty) which necessarily renders a blood vessel site at risk of restenosis.

One preferred type of pharmaceutically acceptable carrier is a polymeric material, such as a hydrogel, that does not cause an adverse or undesired reaction such as inflammation. Many such materials are known, including those made from both natural and synthetic polymers. Many of these polymeric materials are available commercially and have varying properties such as different melting temperatures and biodegradeability profiles, such that a particular pharmaceutically acceptable carrier can be selected to accommodate the specific type of method selected to deliver the multi-G oligonucleotide of the present invention. Generally speaking, the multi-G oligonucleotides are admixed into the selected polymeric material in a liquid state. The resulting mixtur is applied onto the surface to be treated, *e.g.*, by

spraying or painting during surgery or using a catheter or endoscopic procedures.

Other preferred pharmaceutically acceptable carriers for therapeutic administration of the multi-G oligonucleotides of the present invention include lipids, liposomes, microcapsules, implantable devices and cell surface-directed delivery vehicles including those which operate by membrane insertion. For example, one such cell surface-directed device which operates by way of membrane insertion includes a long chain alkyl function (linker) which may be covalently linked to a selected multi-G oligonucleotide in either a bio-releasable or non-releasable form. The linker arm of the resulting conjugate embeds itself in the membrane of living cells, thereby situating the multi-G oligonucleotide in close proximity to the targeted cell. Multi-G oligonucleotides according to the present invention can also be incorporated into implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate, polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof as the implant material is polymerized or solidified or mechanically mixed with the material. In a preferred embodiment, the oligonucleotides are mixed into or applied onto coatings (which may include, *e.g.*, cell-surface directed delivery vehicles or liposomes) for implantable devices such as dextran-coated silica beads, stents, or catheters.

The following examples are provided to aid in the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understo d that modifications can be made in the

procedures set forth, without departing from the spirit of the invention.

Example 1

Synthesis of Oligodeoxynucleotides

All of the oligonucleotides used in the following examples were synthesized using an Applied Biosystems Inc. (Foster City, California) Model 394 DNA synthesizer. Phosphorothioate modified linkages were introduced by oxidizing the phosphite linkage formed during oligonucleotides synthesis with ^{3}H-1,2-benzodithiole-3-1,1-dioxide (Beaucage reagent, Glen Research, Sterling Virginia) instead of the standard iodine oxidation. The four common nucleoside phosphoramidites (*i.e.*, adenine, thymine, guanine and cytosine) and deoxyinosine phosphoramidite were purchased from Applied Biosystems Inc. All unmodified phosphodiester and modified thioate-containing oligonucleotides were deprotected by treatment with concentrated ammonia at 55°C for 12 hours. The oligonucleotides were purified by gel exclusion chromatography and ethanol precipitation and lyophilized to dryness.

Unlike mixed base oligonucleotides, homopolymers of guanosine (*i.e.* poly-G oligonucleotides) are not soluble in 1 ml of water after concentration. In order to solubilize these poly-G compounds, the purified homopolymers must first be dissolved by adding 3 ml of formamide to the aqueous suspension, after which 0.4 ml of 3 M sodium acetate is added, followed by 12 ml of isopropanol. These samples must be stored at -20°C overnight. The resulting precipitate can be collected by centrifugation and dried *in vacuo*. The polyguanosine

oligonucleotides, sodium salt form, will now be soluble in water and can subsequently be treated in the same manner as mixed base oligonucleotides, except that freeze-thawing should be avoided by storing aqueous solutions at 4°C.

The oligonucleotides used in the following examples were all fully modified with phosphorothioate linkages and are summarized in Figure 1. With reference to Figure 1, all 18-mer phosphorothioate-modified oligonucleotides were based on the sequence of *c-myb* codon numbers two through seven. Nucleotides shown in **bold** were switched to generate either: (1) mismatch controls for the rabbit antisense sequence (Set A); (2) various permutations of the human *c-myb* scrambled (G4) control (Set B); or (3) guanine-containing oligonucleotides having inosine substitutions (Set C). The contiguous guanine residues discussed herein are underlined and are indicated parenthetically with the identifying name assigned to the particular oligonucleotide.

Example 2

In vitro Antiproliferative Effects of Multi-G Oligonucleotides Against Rabbit and Pig Smooth Muscle Cells

This example demonstrates a comparison of the inhibitory action of potential antisense oligonucleotides and multi-G oligonucleotides against smooth muscle cell proliferation in an *in vitro assay*. The antisense sequences were designed using the *c-myb* gen as the antisens target, and were compared against multi-G oligonucleotides, scrambled oligonucleotid s and control oligonucleotides. All of the oligonucleotides

in this example were fully modified with phosphorothioate linkages.

Oligonucleotide Design

Specifically, one set of oligonucleotides was based on an antisense sequence against the *c-myb* target, but contained two strategically placed mismatched bases (Set A, Figure 1). It was expected that these mismatches would significantly disrupt hybridization of the oligonucleotide to the target gene/mRNA, thus blocking any anti-proliferative effect caused by a true antisense mechanism. One of the mismatch sequences also disrupts the four contiguous guanine residues that also coincidentally occur in the antisense sequence. In addition, two control sequences were designed which have no known antisense targets. One, rb SCR (random), was designed as true random control oligonucleotide, wherein the rb AS (G4) nucleotide sequence was randomized. Thus, the base composition and length of the oligonucleotide were maintained, but the sequence information was completely scrambled. The second control oligonucleotide, rb SCR (G4), maintained the position and length of the multi-G region (4 Gs within an 18 mer oligonucleotide), but the nucleotides on both the 5'- and 3'-side of the multi-G region were completely scrambled.

A second set of oligonucleotides (Set B, Figure 1) was based on a scrambled multi-G oligonucleotide sequence having four contiguous guanine residues, but with the remainder of the sequence being "scrambled", designated hu SCR (G4). This control sequence had the same base composition as the human anti-*c-myb* antisens sequence. However, the positions of two residues within this sequence w re further

altered to either retain or disrupt the four contiguous guanine residues, in an effort to further investigate the role of multi-G oligonucleotides in inhibition smooth muscle cell growth.

A third set of oligonucleotides (Set C, Figure 1) was designed based upon the hu SCR (G4) control sequence, wherein the guanine residues in the sequence were replaced by either two or four of the structurally related base inosine (I).

The *c-myb* sense sequence was included in the oligonucleotide series for the purpose of serving as an additional control for determining non-specific effects on proliferation. Specifically, the Set A oligonucleotides shown in Figure 1 were assayed for anti-proliferative effect against smooth muscle cells in culture according to the following assay.

In vitro Assay

An *in vitro* smooth muscle cell proliferation assay was performed as follows. Primary rabbit smooth muscle cells were isolated by an explant procedure from the aorta of New Zealand White Rabbits. The cells were expanded in culture (using a growth media of DME Dulbeco's Modified Eagle's medium (Gibco) + 10% FBS fetal bovine serum (HyClone), Penicillin/Streptomycin (Gibco) and glutamine) for 2 passages. Passage 2 ("P2") cells were frozen. The frozen cells were grown up and confirmed to be smooth muscle cells using antibodies to SMC-specific isoforms of actin and myosin. Essentially all of the cells stained positively for both proteins.

All experiments wer performed on P3 cells by thawing P2 cells into growth media, allowing them to grow

just to confluence and then replating them into 12-well culture dishes at 20,000 cells per well. After allowing attachment to take place overnight, the cells were rinsed twice with phosphate buffered saline (PBS), and then placed into a "starvation media" (DME + 0.1% FBS, Pen/Strep and glutamine) for 3 or 4 days to arrest the cells in G_0 of the cell cycle. To initiate proliferation, the starvation media was removed and replaced with normal growth media (10% FBS) with or without completely modified (phosphorothioate-containing) oligonucleotides at the designated concentrations. Triplicate wells for every sample were analyzed. Zero counts were performed at the initiation of proliferation (*i.e.*, on starved cells) to confirm that the cells were growth arrested. (BrdU-labeling experiments also confirmed that the cells were not cycling under these conditions, as described below). Following 3 days of growth, the cells were trypsinized and counted with a Coulter Counter. The percent suppression was calculated according to the following formula:

$$\frac{(F_C - Z_C)\text{control} - (F_C - Z_C)+\text{oligo}}{(F_C - Z_C)\text{control}}$$

wherein Z_C (zero count) is the number of cells per well after the starvation period, and F_C (final count) is the number of cells per well after the 3 day proliferation period.

Results

Figures 2A, 2B and 2C show the percent suppression of rabbit smooth muscle cells at 10, 30 and 60 μM oligonucleotide concentrations, respectively, for each of th above-described oligonucleotides in Set A. Surprisingly, the results of the *in vitro* t sts

demonstrated that the anti-proliferative effect observed with respect to smooth muscle cells is not due to a true antisense mechanism. All of the multi-G oligonucleotides were significantly more inhibitory than the three sequences (designated with an "*" in Figures 2A, 2B and 2C) lacking the four contiguous guanine residues. The most interesting result observed was the decreased anti-proliferative effect of the rb AS 6/12 oligonucleotide sequence, wherein the four contiguous guanine residues were disrupted. Similar mismatch sequences rb AS 4/12 (G4) and rb AS 2/18 (G4) inhibited proliferation to an extent similar to the antisense and other multi-G oligonucleotides. The anti-proliferative effect of the multi-G oligonucleotides became less important at higher oligonucleotide concentrations, which was probably due to a general anti-proliferative effect caused by the high concentration of sulfur-containing modified oligonucleotides.

The same experiments were repeated with pig smooth muscle cells, using the highly homologous rabbit antisense and multi-G oligonucleotide sequences (Set A in Figure 1), identified above. Figure 2D shows remarkably similar results in pig smooth muscle cells, wherein all of the multi-G oligonucleotides were considerably more inhibitory than the control sequences which lack four contiguous guanine residues (designated with an "*" in Figure 2D).

Example 3

Analysis Scrambled Multi-G Oligonucleotides

In order to demonstrate that inhibition by the initially id ntified rb AS (G4) multi-G oligonucleotide was due to th four contiguous guanine residues, a

series of additional sequences was designed which did not correspond to any known antisense sequence. The overall base composition of these oligonucleotides was identical to the hu AS (G4) sequence, but the position of two nucleotides was switched, such that the resulting oligonucleotides would either preserve or disrupt the four contiguous guanine residues. The specific sequences tested in this example (*see*, Set B in Figure 1) were as set forth in Table I:

Table I

Oligonucleotide	Sequence	SEQ ID NO
hu SCR (G4)	GCT GTG GGG CGG CTC CTG	11
hu SCR 10/17 (G4)	GCT GTG GGG TGG CTC CCG	12
hu SCR 10/18 (G7)	GCT GTG GGG GGG CTC CTC	13
hu SCR 6/13 (2xG3)	GCT GTC GGG CGG GTC CTG	14
hu SCR 8/15 (2xG2)	GCT GTG GCG CGG CTG CTG	15

These oligonucleotides were analyzed for inhibition of smooth muscle cell proliferation according to the *in vitro* assay described in Example 2. The results, shown in Figure 3, show that inhibition by this class of oligonucleotides is due to the presence of either four contiguous guanine residues or two series of three contiguous guanine residues. However, disruption of the four contiguous guanine residues [hu SCR (random) and hu SCR 8/15 (2xG2)] was observed to abrogate the inhibition of smooth muscle cell proliferation. In addition, it was observed that the hu SCR 10/18 (G7) sequence was even more inhibitory than the oligonucleotides having only four contiguous guanine residues, presumably due to the longer length of the contiguous guanine residue series; *i.e.*, seven as compared to four. *See also*, Examples 5 and 6, below.

Example 4

Analysis of Inosine-Containing Oligonucleotides

This example demonstrates the decrease in anti-proliferative effect that is observed when a series contiguous guanine residues in a multi-G oligonucleotide is disrupted. Because it was desired to change as little as possible with respect to base composition of the oligonucleotides, inosine was selected to create the desired disruption in the series of contiguous guanine residues.

Although it is structurally very similar to guanine, inosine is an extremely rare base that only appears in naturally occurring DNA or RNA at a rate of approximately one in every 1,000 bases. Unlike guanine, however, inosine cannot participate to the same degree in hydrogen bonding, because it lacks the 2-amino functionality which is present in guanine. Because the results from Example 2 suggested a relationship between the observed anti-proliferative effect and the four contiguous guanine residues in the multi-G oligonucleotides, three additional oligonucleotides were designed in which either 2 or 4 of the guanine residues in the series of contiguous guanine residues were replaced with inosine nucleotides. The sequence of the hu SCR (G4) multi-G oligonucleotide was otherwise maintained. The particular sequences used in this example, set forth in Table II below (*see*, Set C in Figure 1), were analyzed for anti-proliferative activity against smooth muscle cells according to the *in vitro* assay described in Example 2.

Table II

Oligonucleotide	Sequence	SEQ ID NO
hu SCR (G4)	GCT GTG GGG CGG CTC CTG	11
hu SCR (I2G2)	GCT GTI IGG CGG CTC CTG	16
hu SCR (G2I2)	GCT GTG GII CGG CTC CTG	17
hu SCR (I4)	GCT GTI III CGG CTC CTG	18

Comparison of the hu SCR multi-G oligonucleotides to the inosine-substituted oligonucleotides at 30 μM is shown in Figure 4. The data demonstrated a clear decrease in the level of suppression of smooth muscle cell proliferation when either 2 or 4 of the guanine residues were substituted with inosine. However, the level of inhibition of smooth muscle cell proliferation observed for the inosine-substituted oligonucleotides was still more pronounced than that seen with the scrambled oligonucleotide sequences from Example 2 (around 20% at 30 μM), suggesting that there is partial inhibition from the effect observed for contiguous guanine sequences, even when the contiguous guanine sequence is substituted with inosine residues. Nevertheless, this example confirms that the antiproliferative effect observed in Example 2 is the result of a sequence specific, non-antisense, effect caused by the presence of multiple contiguous guanine residues.

Example 5

In vitro Screening of Multi-G Oligonucleotide Pools

Onc it was confirmed that the anti-proliferative effect observed against smooth muscle cells was caused by the presence of the contiguous

guanine residues in multi-G oligonucleotides, it was desired to screen additional multi-G oligonucleotides through *in vitro* testing to determine particular optimal multi-G oligonucleotides. To this end, random sequences from a "G4 pool" were screened for *in vitro* suppression of smooth muscle cell proliferation according to the *in vitro* assay described in Example 2.

Specifically, the "G4 pools" were generated by using the following 18-mer multi-G sequence as a template from which to build randomized oligonucleotides:

5' NNN NNG GGG XNN NNN NNG 3'

wherein "N" designates a randomized position (representing an equal mixture of A, G, C or T), and X indicates a fixed position wherein a single residue of A, G, C or T was incorporated. Accordingly, the following four pools of multi-G oligonucleotides, set forth in Table III were made according to the oligonucleotide synthesis method of Example 1.

Table III

Oligonucleotide	Sequence	SEQ ID NO
G4/T	NNN NNG GGG TNN NNN NNG	19
G4/C	NNN NNG GGG CNN NNN NNG	20
G4/A	NNN NNG GGG ANN NNN NNG	21
G4/G	NNN NNG GGG GNN NNN NNG	22

As in the previous examples, all of the oligonucleotides in the above-described G4 pools were completely modified with phosphorothioate linkag s. The anti-proliferative effect of these oligonucleotides was determined according to the *in vitro* anti-proliferation

assay described in Example 2, with each of the G4/T, G4/C, G4/A and G4/G pools being assayed a minimum of seven times. The results are shown in Table IV below:

Table IV

	% Suppression*			
Oligonucleotide	10 μM	5 μM	2 μM	1 μM
G4/A Pool	44	19	--	1
G4/C Pool	57	30	--	-3
G4/T Pool	57	20	--	0
G4/G Pool	76	63	33	12

*Average of all runs

At 5 μM and 10 μM oligonucleotide concentrations, the G4/G pool, having five contiguous guanine residues, demonstrated the greatest amount of inhibition against smooth muscle cell proliferation, particularly at the 5 μM level, indicating that these five contiguous guanine residues may be preferred over four contiguous guanine residues for inhibition of smooth muscle cell growth in certain situations.

Multi-G oligonucleotide pools were also generated for oligonucleotides having: (1) five contiguous guanine residues; and (2) six contiguous guanine residues. The pools in this example were generated in a manner similar to the pools described above for multi-G oligonucleotides having four contiguous guanine residues, except that the pools for multi-G oligonucleotides having five contiguous guanine residues were made with a 3' defined base, according to the formula: $(N)_4G_5X(N)_7G$; and the pools for multi-G oligonucleotides having six contiguous guanine residues

were made with a 5' defined base, according to the formula: $(N)_3XG_6(N)_7G$. Once again, all of the multi-G oligonucleotides made in this example were fully modified with phosphorothioate linkages and were tested in the *in vitro* smooth muscle cell proliferation assay described in Example 2, with the G5 pool oligonucleotides being assayed a minimum of five times and the G6 pool oligonucleotides being assayed a minimum of three times. The results are shown in Tables V (G5 pool) and VI (G6 pool) below:

Table V

	% Suppression*			
Oligonucleotide	10 µM	5 µM	2 µM	1 µM
G5/A Pool	--	54	--	21
G5/C Pool	--	63	--	29
G5/T Pool	--	60	--	43
G5/G Pool	--	73	52	50

*Average of all runs

At 5 µM and 1 µM oligonucleotide concentrations, the G5/G pool, having six contiguous guanine residues, demonstrated the greatest amount of inhibition against smooth muscle cell proliferation, similar to the G4 pool results, except that the difference in inhibition of the different G5 pools at the level was not nearly as marked as was observed in the case of the G4 pools.

Table VI

Oligonucleotide	% Suppression* 10 μM	5 μM	2 μM	1 μM
G6/A Pool	--	70	--	--
G6/C Pool	--	63	--	--
G6/T Pool	--	66	--	--
G6/G Pool	--	72	--	--

*Average of all runs

At 5 μM oligonucleotide concentrations, all of the G6 pools demonstrated more comparable levels of inhibition, although, the G6/G pool, having seven contiguous guanine residues, was still found to have the greatest inhibitory effect against smooth muscle cell proliferation, especially at lower concentrations (data not shown in Table VI.)

Example 6

In vitro Analysis of Multi-G Oligonucleotides Having Additional Contiguous Guanine Residues

This example demonstrates the varying degrees of inhibition observed for a variety of multi-G oligonucleotides having additional contiguous guanine residues to determine optimal multi-G residues for use in the inhibition of smooth muscle cell proliferation. Specifically, the following fully modified (phosphorothioate) 18-mer oligonucleotides, set forth in Table VII, below, were made according the procedures described in Example 1.

Table VII

Oligonucleotide	Sequence	SEQ ID NO.
T7-G4-T7	TTT TTT TGG GGT TTT TTT	23
T5-G8-T5	TTT TTG GGG GGG GTT TTT	24
T3-G12-T3	TTT GGG GGG GGG GGG TTT	25
T-G16-T	TGG GGG GGG GGG GGG GGT	26
T4-G4-T2-G4-T4	TTT TGG GGT TGG GGT TTT	27
A4-G4-A2-G4-A4	AAA AGG GGA AGG GGA AAA	28
T3-G4-T4-G4-T3	TTT GGG GTT TTG GGG TTT	29
A3-G4-A4-G4-A3	AAA GGG GAA AAG GGG AAA	30
T2-G4-T6-G4-T2	TTG GGG TTT TTT GGG GTT	31
A2-G4-A6-G4-A2	AAG GGG AAA AAA GGG GAA	32
T-G4-T8-G4-T	TGG GGT TTT TTT TGG GGT	33
A-G4-A8-G4-A	AGG GGA AAA AAA AGG GGA	34

These multi-G oligonucleotides were then assayed for inhibition against smooth muscle cell proliferation according to the procedures described in Example 2. The results are shown below in Table VIII, below.

Table VIII

Oligonucleotide	% Suppression* 5 μM	2 μM	1 μM	0.5 μM
T7-G4-T7	3	-12	--	--
T5-G8-T5	72	43	--	--
T3-G12-T3*	86	69	--	--
T3-G12-T3**	--	67	48	23
T-G16-T*	81	64	--	--
T-G16-T**	--	77	49	32
T4-G4-T2-G4-T4	58	22	--	--
A4-G4-A2-G4-A4	47	-2	--	--
T3-G4-T4-G4-T3	49	9	--	--
A3-G4-A4-G4-A3	54	-1	--	--
T2-G4-T6-G4-T2	63	20	--	--
A2-G4-A6-G4-A2	59	20	--	--
T-G4-T8-G4-T	56	1	--	--
A-G4-A8-G4-A	40	-6	--	--

*First run

**Second run

These results show that multi-G oligonucleotides having longer series of contiguous guanine residues, *e.g.*, twelve and 16 guanine residues, demonstrate a higher degree of inhibition against smooth muscle cell proliferation than multi-G oligonucleotides with four contiguous guanine residues, even where two series of four contiguous guanine residues were contained within the oligonucleotide. This difference was particularly magnified at the 2 μM concentration range, indicating that a higher degree of inhibition can be achieved at lower oligonucleotide concentration ranges if eight or more contiguous guanin residues are incorporated into the oligonucleotide.

Example 7

Mechanism of Action of Multi-G Oligonucleotides

In order to determine the mechanism by which multi-G oligonucleotides inhibit smooth muscle cell proliferation, two experiments were conducted. In the first experiment, incorporation of bromodeoxyuridine (BrdU) was measured. In the second experiment, recovery of smooth muscle cells following treatment with multi-G oligonucleotides was determined.

BrdU Incorporation

Smooth muscle cells were treated according to the standard *in vitro* proliferation assay described in Example 2, with the following exceptions: (1) the cells were plated onto *Nunc* culture slides; and (2) following the starvation period, 10% serum and BrdU were added simultaneously and left in contact with the cells for approximately 24 hours. The level of BrdU incorporation was detected using a horseradish peroxidase (HRP)-linked secondary antibody to BrdU, followed by an HRP-dependent staining reaction. Total nuclei were labeled by incubating fixed cells with propidium iodide. An image analysis program was developed to automatically count the BrdU labeled nuclei and the total nuclei based on their different staining.

Figure 5 shows the results of the BrdU incorporation assay. The pattern of inhibition seen in the BrdU experiment is remarkably similar to the results obtained using the cell count assay (Examples 2 and 3). This confirms that the multi-G oligonucleotides of the present invention are preventing ntry into the cell

cycle, rather than exerting a toxic effect on smooth muscle cells.

Recovery of Smooth Muscle Cells Following Treatment

Smooth muscle cells were subjected to treatment with multi-G oligonucleotides at 30 μM concentrations. At the end of 3 days, one set of wells was counted to determine the percent suppression (as measured in a typical assay). Identically treated wells were washed once with PBS and then fed with normal growth media. One set of wells was counted on each subsequent day for 3 days to determine if the smooth muscle cells could recover from treatment with oligonucleotides and proliferate normally following removal of the oligonucleotide.

Removal of the multi-G oligonucleotide led to a complete recovery of the smooth muscle cells, as shown in Figure 6. Specifically, the smooth muscle cells began to proliferate normally and continued growing until they reached confluence (in 3 days) at which time they stopped growing due to a typical density arrest. The growth rate of the multi-G oligonucleotide-treated cells during recovery was identical to control cells which were released from serum deprivation.

Results

Both the BrdU incorporation data and the recovery experiment described above demonstrate that smooth muscle cell cultures treated with multi-G oligonucleotides are prevented from proliferating by a non-toxic blockage of their ability to re-enter the cell cycle after growth arrest in G_0. The fact that th inhibitory effect of the multi-G oligonucleotides is

fully reversible upon removal of the oligonucleotide from the culture media indicates that the observed inhibition of smooth muscle cell proliferation is due to an inhibitory action other than general toxicity of the oligonucleotides.

Example 8

Ex vivo Antiproliferative Effects of Multi-G Oligonucleotides

The purpose of this example was to confirm that the antiproliferative effects observed for multi-G oligonucleotides on smooth muscle cells *in vitro* correlates to an observed antiproliferative effect in arteries *ex vivo*. The sequences used in this example were previously identified in Example 2 as having a strong *in vitro* antiproliferative effect.

According to the *ex vivo* assay system employed in this example, crush-damaged ear artery segments of rabbits were incubated (*ex vivo*) in various multi-G oligonucleotide solutions, with proliferation being evaluated by incorporation of a thymidine analog label, namely bromodeoxyuridine (BrdU) into smooth muscle cell nuclei. A rabbit ear artery model was used, because injury response in this tissue is approximately the same in this *ex vivo* system as it is *in vivo*. In either case (*i.e., in vivo* or *ex vivo*) there is induction of proliferation of medial smooth muscle cells that appears maximal at 72 hours after the initial injury.

Injury

To induce crush injury in the rabbit ear artery, the rabbits were deeply anesthetized, aft r

which 12 mm acrylic discs were centered over the central vascular bundle (which contains the central artery) of both sides of each ear and secured with Kelly clamp forceps. After 30 minutes, the forceps were released, the discs removed, and the rabbit terminated by lethal injection. The length of vascular bundle under the discs was dissected free of surrounding tissue, rinsed in phosphate buffered saline and removed to culture. A total of 72 artery segments were obtained from 36 rabbits for use in these experiments.

Treatments

The rabbit ear arteries were incubated in wells of a 24-well culture dish containing DMEM + 5% FBS and the appropriate treatment. A total of seven different oligonucleotides were screened in three separate experiments using groups of 12 rabbits per experiment. The 24 artery segments from the 12 rabbits were randomized into one of three oligonucleotides treatments (at 100 μM) or control per experiment. The oligonucleotides sequences screened were as follows:

Experiment A:	rb AS (G4)
	hu SCR (G4)
	rb SCR (random)
Experiment B:	rb AS (G4)
	rb AS 4/12 (G4)
	rb AS 6/12 (G3)
Experiment C:	hu SCR (G4)
	hu SCR 6/13 (2xG3)
	hu SCR 8/15 (2xG2)

Proliferation Assay

At 68 hours after injury, BrdU was added to each of the culture wells at a final concentration of 10 µg/ml, and the vascular bundle segments incubated for a further 4 hours. At 72 hours the media was removed, the samples rinsed in PBS and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 4 hours. The samples were then dehydrated through a series of alcohols and processed into paraffin blocks. The segments were serial sectioned and the sections stained with antibodies to BrdU . After immunostaining on a TechMate immunostainer (BioTek Solutions Inc., Santa Barbara, California) the sections were fitted with coverslips and the number BrdU-labeled medial smooth muscle cells quantified using a Quantimet 520 Image Analyzer (Leica, Cambridge, Massachusetts). The medial area and the number of nuclei in that area were measured in 20 serial sections, totaled and used to calculate a single density measurement for each segment according to the following formula:

Total number of nuclei/ Total area = Number nuclei/mm^2

The individual measures for each artery were ranked and median value presented in the graphs shown in Figures 7A (Experiment A), 7B (Experiment B) and 7C (Experiment C).

Results

The trends illustrated in the graphs of the three experiments follow the trends that were elucidated in the *in vitro* assays. Incubation of the artery in the rb AS (G4), and 2xG3 oligonucl otides resulted in substantial reduction in the number of BrdU-labeled

nuclei in the media compared to the untreated, control arteries and compared to the arteries treated with the other oligonucleotides.

This shows that the phosphorothioate-modified scrambled rb AS (G4) oligonucleotide potently inhibits the proliferation of smooth muscle cells in arteries *ex vivo*, and further supports the results presented in Examples 2 and 3 wherein a sequence specific anti-proliferative effect was observed *in vitro* that was not due to a true antisense mechanism.

This data further supports the evidence that the multi-G oligonucleotides identified *via* the *in vitro* screening process inhibit smooth muscle cell proliferation in the arterial wall. It is expected that inhibition of this proliferation will reduce or eliminate the formation of the neointima in angioplastied arteries that eventually results in the clinical reclosure of arteries called restenosis.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Burgess, Teresa L.
Farrell, Catherine L.
Fisher, Eric F.

(ii) TITLE OF INVENTION: Method for Inhibiting Smooth Muscle Cell Proliferation and Oligonucleotides for Use Therein

(iii) NUMBER OF SEQUENCES: 34

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette, 3.5 in., DS, 1.4 MB

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(A) APPLICATION NUMBER: 08/041,746

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(C) CLASSIFICATION: not yet known

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTGCCGGGGT CTCCGGGC 18

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCCGTCGCG GCGGTTGG 18

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GCCCGGAGAC CCCGGCAC 18

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGTCGGGGT CTCCGGGC 18

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTGCGGGGC GGCTCCTG 18

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCGCCGGGGT CTCCGGGT 18

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTGTCGGGGT CCCCGGGC 18

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTGCCTGGGT CGCCGGGC 18

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTGCCGGGGT CTTCGGGC 18

(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TGCCTGCGCG GCGGTTGG 18

(12) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCTGTGGGGC GGCTCCTG 18

(13) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTGTGGGGT GGCTCCCG 18

(14) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCTGTGGGGG GGCTCCTC 18

(15) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCTGTCGGGC GGGTCCTG 18

(16) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTGTGGCGC GGCTGCTG 18

(17) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCTGTIIGGC GGCTCCTG 18

(18) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GCTGTGGIIC GGCTCCTG 18

(19) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GCTGTIIIIC GGCTCCTG 18

(20) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:19:

NNNNNGGGGT NNNNNNNG 18

(21) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:20:

NNNNNGGGGC NNNNNNNG 18

(22) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: lin ar

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:21:

NNNNNGGGGA NNNNNNNG 18

(23) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:22:

NNNNNGGGGG NNNNNNNG 18

(24) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TTTTTTTGGG GTTTTTTT 18

(25) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TTTTTGGGGG GGGTTTTT 18

(26) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TTTGGGGGGG GGGGGTTT 18

(27) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TGGGGGGGGG GGGGGGGT 18

(28) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TTTTGGGGTT GGGGTTTT 18

(29) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AAAAGGGGAA GGGGAAAA 18

(30) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTTGGGGTTT TGGGGTTT 18

(31) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AAAGGGGAAA AGGGGAAA 18

(32) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TTGGGGTTTT TTGGGGTT 18

(33) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:32:

AAGGGGAAAA AAGGGGAA 18

(34) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:33:

TGGGGTTTTT TTTGGGGT 18

(35) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 bases

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AGGGGAAAAA AAAGGGGA 18

What is claimed is:

1. A modified oligonucleotide from about 10 to about 30 bases in length having at least two series of at least four contiguous guanine residues.

2. The modified oligonucleotide of claim 1 wherein said modified oligonucleotide has at least one residue other than a guanine residue at each of the 3'- and 5'-ends of said oligonucleotide.

3. The modified oligonucleotide of claim 2 wherein said oligonucleotide has at least one base other than a guanine residue between said two series of at least four contiguous guanine residues.

4. The modified oligonucleotide of claim 3 wherein said modified oligonucleotide has at least two series of at least six contiguous guanine residues.

5. The modified oligonucleotide of claim 4 wherein said modified oligonucleotide is from about 14 to about 20 bases in length.

6. The modified oligonucleotide of claim 5 wherein said modified oligonucleotide is an 18-mer.

7. The modified oligonucleotide of claim 2 wherein said two series of contiguous guanine residues are adjacently situated in said oligonucleotide such that said modified oligonucleotide has at least eight contiguous guanine residues.

8. The modified oligonucleotide of claim 7 wherein said modified oligonucleotide has at least two series of at least six contiguous guanine residues.

9. The modified oligonucl otide of claim 8 wherein said modified oligonucleotide is from about 14 to about 20 bases in length.

10. The modified oligonucleotide of claim 9 wherein said modified oligonucleotide is an 18-mer.

11. The modified oligonucleotide of claim 10 wherein said modified oligonucleotide has a series of 8 to 16 contiguous guanine residues.

12. The modified oligonucleotide of claim 11 wherein said oligonucleotide has a series of contiguous guanine residues selected from the group consisting of 8 contiguous guanine residues, 12 contiguous guanine residues and 16 contiguous guanine residues.

13. A method for inhibiting the proliferation of smooth muscle cells comprising contacting said smooth muscle cells with a modified multi-G oligonucleotide from about 10 to about 30 bases in length.

14. The method of claim 13 wherein said modified multi-G oligonucleotide has a residue other than a guanine residue at each of the 3'- and 5'-ends of said modified multi-G oligonucleotide.

15. The method of claim 14 wherein said modified multi-G oligonucleotide has two series of three contiguous guanine residues separated by a single residue other than a guanine residue.

16. The method of claim 14 wherein said modified multi-G olig nucleotide has a singl series four contiguous guanin residues.

17. The method of claim 14 wherein said modified multi-G oligonucleotide has at least two series of at least four contiguous guanine residues.

18. The method of claim 17 wherein said two series of at least four contiguous guanine residues are separated by at least one residue other than a guanine residue.

19. The method of claim 17 wherein said at least two series of contiguous guanine residues are adjacently situated in said modified multi-G oligonucleotide such that said modified oligonucleotide has at least eight contiguous guanine residues.

20. The method of claim 19 wherein said modified multi-G oligonucleotide has at least two series of at least six contiguous guanine residues.

21. A pharmaceutical composition comprising a modified multi-G oligonucleotide from about 10 to about 25 bases in length in combination with a pharmaceutically acceptable delivery vehicle.

22. The pharmaceutical composition of claim 21 wherein said modified multi-G oligonucleotide has a residue other than a guanine residue at each of the 3'- and 5'-ends of said modified multi-G oligonucleotide.

23. The pharmaceutical composition of claim 22 wherein said modified multi-G oligonucleotide has two series of three contiguous guanine residues separated by a single residue other than guanine.

24. Th pharmaceutical composition of claim 22 wherein said modified multi-G oligonucleotide has a single series four contiguous guanine residues.

25. The pharmaceutical composition of claim 22 wherein said modified multi-G oligonucleotide has at least two series of at least four contiguous guanine residues.

26. The pharmaceutical composition of claim 23 wherein said two series of at least four contiguous guanine residues are separated by at least one residue other than a guanine residue.

27. The pharmaceutical composition of claim 25 wherein said at least two series of contiguous guanine residues are adjacently situated in said modified multi-G oligonucleotide such that said modified oligonucleotide has at least eight contiguous guanine residues.

28. The pharmaceutical composition of claim 27 wherein said modified multi-G oligonucleotide has at least two series of at least six contiguous guanine residues.

29. The pharmaceutical composition of claim 21 wherein said oligonucleotide in combination with said pharmaceutically acceptable delivery vehicle is incorporated onto the surface of a catheter or stent.

30. A method for screening multi-G oligonucleotides for use in the inhibition of vascular rest nosis comprising the steps of:

a) arresting smooth muscle c lls in culture in G_0 growth phas ;

b) initiating proliferation of said smooth muscle cells in the presence of multi-G oligonucleotides; and

c) selecting the multi-G oligonucleotides which demonstrate greater than about 50% inhibition at a concentration of less than or equal to 10 μM.

31. The method of claim 29 wherein said smooth muscle cells are attached to a solid support.

32. The method of claim 29 wherein said smooth muscle cells are arrested in G_0 growth phase by placing said smooth muscle cells in a starvation media after which proliferation of said smooth muscle cells is initiated by replacing said starvation media with normal growth media.

33. The method of claim 29 wherein said multi-G oligonucleotides are selected on the basis of demonstrating greater than about 50% inhibition at a concentration of less than or equal to 2 μM.

FIGURE 1

Oligonucleotide	Sequence	SEQ ID NO
Set A:		
rb AS (G4)	GTG CCG GGG TCT CCG GGC	1
rb SCR (random)	CGC CGT CGC GGC GGT TGG	2
rb sense	GCC CGG AGA CCC CGG CAC	3
mu AS (G4)	GTG **T**CG GGG TCT CCG GGC	4
rb SCR (G4)	GCT GCG GGG CGG CTC CTG	5
rb AS 2/18 (G4)	G**C**G CCG GGG TCT CCG GG**T**	6
rb AS 4/12 (G4)	GTG **T**CG GGG TC**C** CCG GGC	7
rb AS 6/12 (G3)	GTG CC**T** GGG TC**G** CCG GGC	8
Set B:		
hu AS (G4)	GTG CCG GGG TCT TCG GGC	9
hu SCR (random)	TGC CTG CGC GGC GGT TGG	10
hu SCR (G4)	GCT GTG GGG CGG CTC CTG	11
hu SCR 10/17 (G4)	GCT GTG GGG **T**GG CTC C**C**G	12
hu SCR 10/18 (G7)	GCT GTG GGG **G**GG CTC CT**C**	13
hu SCR 6/13 (2xG3)	GCT GT**C** GGG CGG **G**TC CTG	14
hu SCR 8/15 (2xG2)	GCT GTG G**C**G CGG CT**G** CTG	15
Set C:		
hu SCR (G4)	GCT GTG GGG CGG CTC CTG	16
hu SCR (I2G2)	GCT GT**I** **I**GG CGG CTC CTG	17
hu SCR (G2I2)	GCT GTG G**II** CGG CTC CTG	18
hu SCR (I4)	GCT GT**I** **III** CGG CTC CTG	19

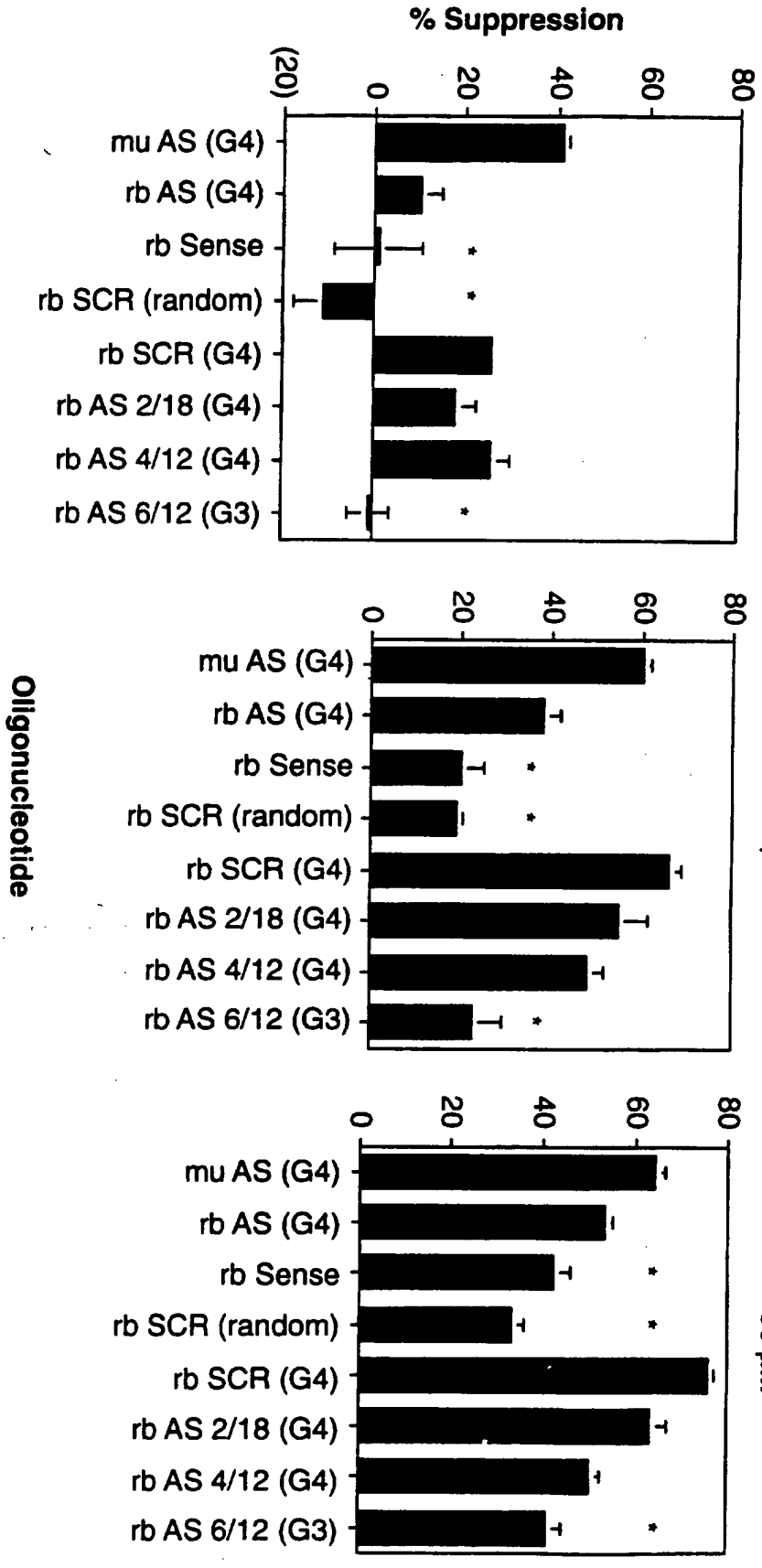


FIGURE 2A

FIGURE 2B

FIGURE 2C

Suppression of Rabbit SMC proliferation by antisense c-myb phosphorothioate oligonucleotides

FIGURE 2D

Suppression of Pig SMC proliferation by antisense c-myb phosphorothioate oligonucleotides

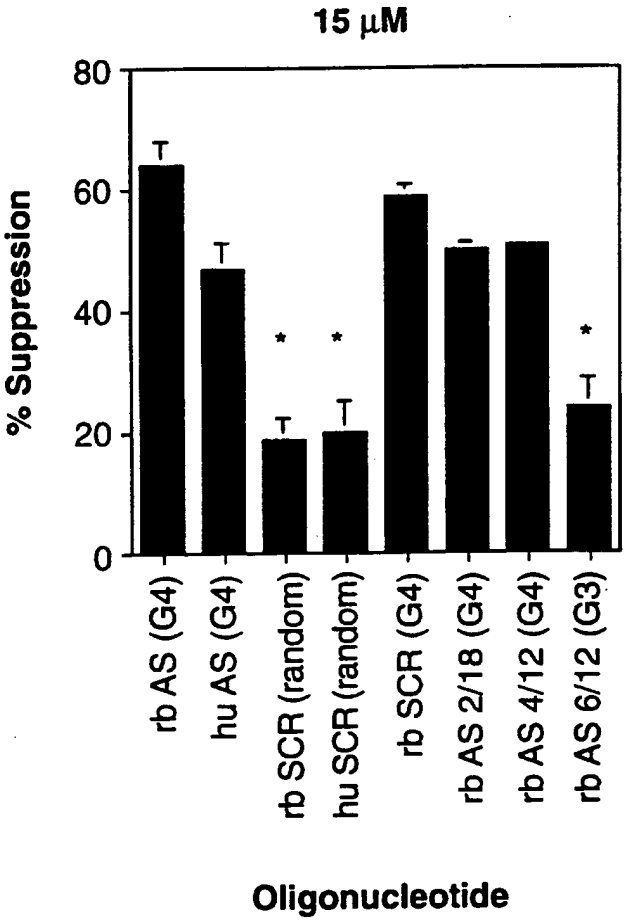


FIGURE 3

Suppression of Rabbit SMC Proliferation by G4 and other control phosphorothioate oligonucleotides

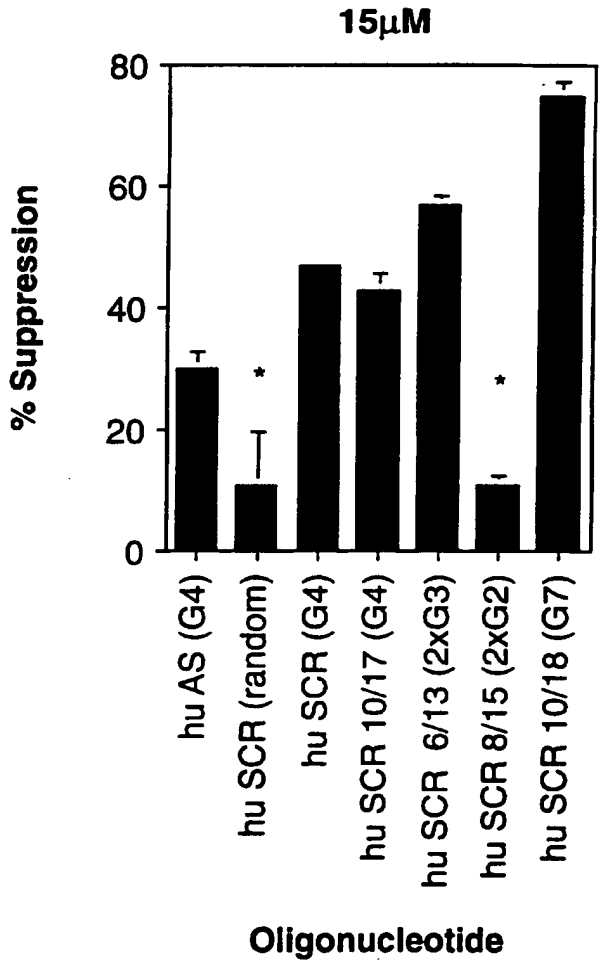


FIGURE 4

Suppression of Rabbit SMC proliferation by Inosine substituted G4 phosphorothioate oligonucleotides

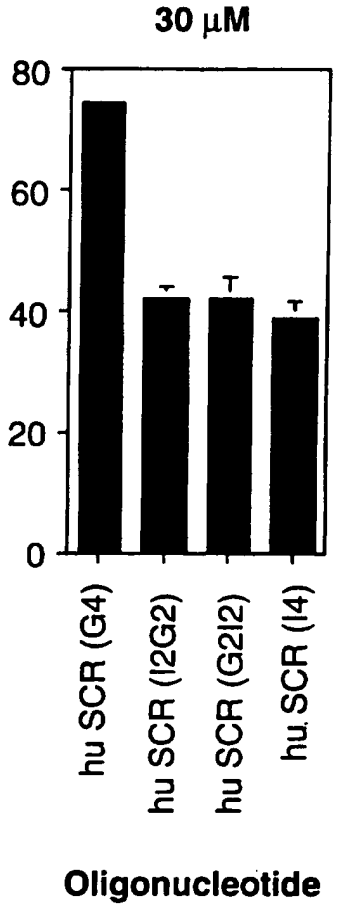


FIGURE 5

Suppression of BrdU incorporation by Rabbit SMCs treated with phosphorothioate oligonucleotides

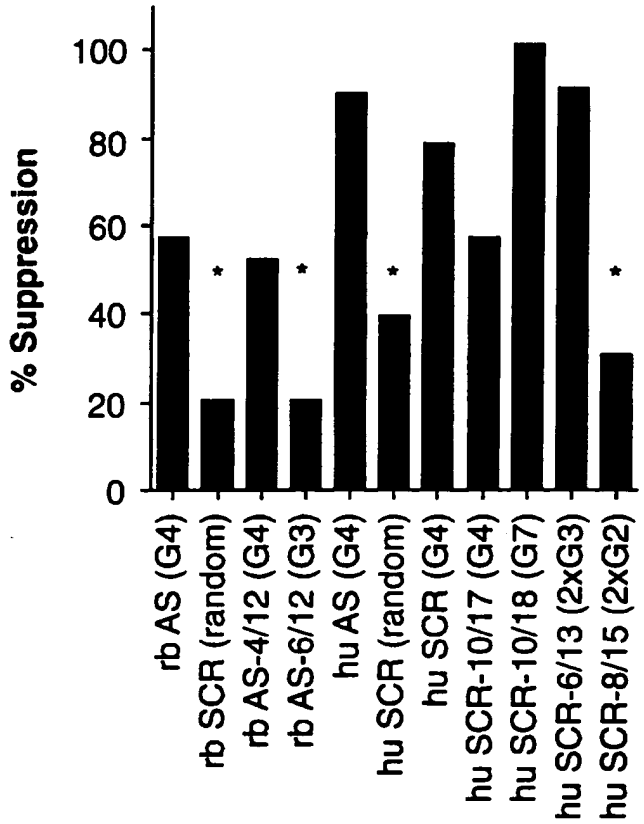
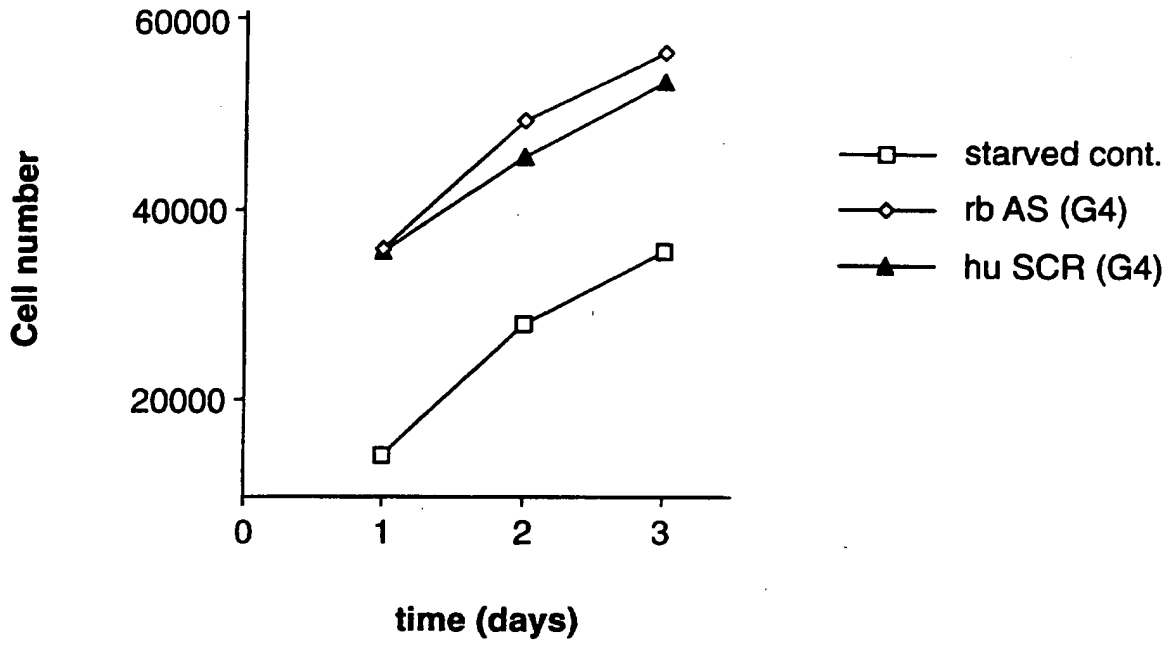
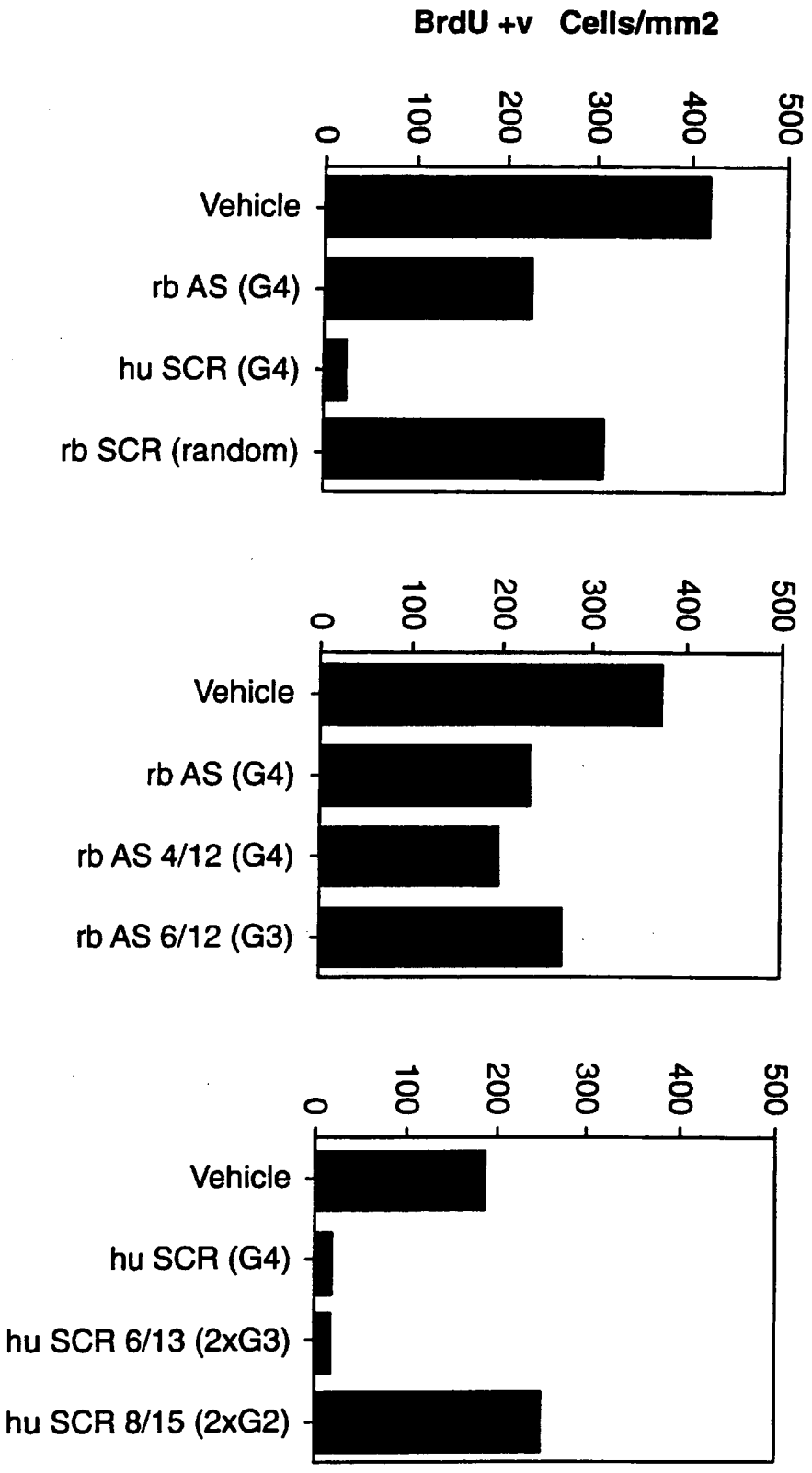


FIGURE 6

Recovery of Rabbit SMC after treatment with phosphorothioate oligonucleotides





Proliferation of SMCs in Rabbit Arteries

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(71) Applicant *(for all designated States except US)*: AMGEN INC. [US/US]; Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: BURGESS, Teresa, L. [-/US]; 908 High Point Drive, Ventura, CA (US). FARRELL, Catherine, L. [-/US]; 28051 Magic Mountain Lane, Canyon Country, CA (US). FISHER, Eric, F. [-/US]; 4188 Amber Place, Boulder County, CO (US).

(74) Agents: ODRE, Steven, M. et al.; Amgen Inc., Amgen Center, 1840 Dehavilland Drive, Thousand Oaks, CA 91320-1789 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).

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(54) Title: METHOD FOR INHIBITING SMOOTH MUSCLE CELL PROLIFERATION AND OLIGONUCLEOTIDES FOR USE THEREIN

(57) Abstract

The present invention provides multi-G oligonucleotides that are capable of inhibiting smooth muscle cell proliferation. The multi-G oligonucleotides of the present invention can be used as nucleic acid therapeutic compounds to inhibit smooth muscle cell proliferation in arteries *ex vivo*, and more particularly against restenosis. The present invention further provides pharmaceutical compositions containing the multi-G oligonucleotides. A method for screening oligonucleotides for their ability to inhibit smooth muscle cell proliferation *ex vivo* is also provided.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/12770

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C07H21/00 A61K31/70 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ANTISENSE RESEARCH AND DEVELOPMENT, vol. 3, no. 1, 1993, US, pages 67-77, XP002015214 YASWEN, P. ET AL.: "Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells" cited in the application see page 72, paragraph 2 - page 74, line 2	1-3
Y	see table 1A	13-18, 21-26,29

X	WO,A,93 01286 (MASSACHUSETTS INST TECHNOLOGY) 21 January 1993 cited in the application see page 17, line 1 - line 30	13-16, 21-24,29
Y	see page 21, SEQ IDs 1 and 4	13-18, 21-26,29

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 1996	28. 10. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Andres, S

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 08053 (ISIS PHARMACEUTICALS INC ;HANECAK RONNIE C (US); ANDERSON KEVIN P) 14 April 1994 cited in the application see page 10, line 12 - page 12, line 6 see page 18, line 25 - line 30 see page 19, line 9 - line 26 see tables 2,3 see page 27, line 23	1-3
A	see examples 19,23,24,27	30-33
X	NUCLEIC ACIDS RESEARCH, vol. 21, no. 8, 25 April 1993, OXFORD GB, pages 1853-1856, XP002015215 ECKER, D. ET AL.: "Rational screening of oligonucleotide combinatorial libraries for drug discovery"	1,2
A	see the whole document	30-33
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 92, no. 9, 25 April 1995, WASHINGTON US, pages 4051-4055, XP002015216 BURGESS, T. ET AL.: "The antiproliferative activity of c-myb and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism" see the whole document	13-16, 30-33
T	ANTISENSE RESEARCH AND DEVELOPMENT, vol. 5, 1995, US, pages 175-183, XP002015217 FARRELL, C. ET AL.: "The uptake and distribution of phosphorothioate oligonucleotides into vascular smooth muscle cells in vitro and in rabbit arteries" see the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12770

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-20
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/12770

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